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Seroepidemiology of Human Group C Rotavirus in the UK

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The gene coding for the major inner capsid protein VP6 of human group C rotavirus was cloned into baculovirus using the pBlueBac2™ vector and expressed in insect cells. When cultured in High Five™ cells, VP6 was expressed at a high level and exported to the cell culture medium. Purified VP6 was used to immunise rabbits. Hyperimmune rabbit serum, which reacted with native human group C rotavirus in infected cells, was used to develop and optimise an EIA for the detection of antibodies to group C rotavirus using the recombinant VP6 as a source of antigen. In a local epidemiological survey of 1000 sera grouped by age, an average of 43% of samples were found to have antibodies to human group C rotavirus with the highest proportion (66%) in the 71–75 year age group. In comparison, 97% of adults and 85% of children had antibodies to recombinant VP6 from the bovine RF strain of group A rotavirus. These results suggest that infection with human group C rotavirus is a common occurrence despite the apparent rarity of reports of human group C rotavirus in clinical samples from patients with gastroenteritis. ***J. Med. Virol. 52:86–91, 1997.***

KEY WORDS: VP6; baculovirus; ELISA; gastroenteritis

INTRODUCTION

Rotaviruses, one of nine genera belonging to the family *Reoviridae,* were first identified as an important cause of gastroenteritis in 1973 [Bishop et al., 1973; Flewett et al., 1973]. Intact virions are 70 nm in diameter and possess an icosahedral double layered protein capsid surrounding a core containing 11 segments of double stranded genomic RNA. Each dsRNA segment codes for a single viral protein ranging in size from 20 to 125 kDa [Estes, 1996].

Rotaviruses are subdivided into 7 serogroups (A–G) on the basis of their antigenic and genetic properties [Kapikian et al., 1996]. Members of each serogroup share a common group antigen located on the major inner capsid protein VP6. Group A rotaviruses are further classified according to subgroup and serotype. The subgroup-specific antigenic determinants are also located on the major inner capsid protein. Serotype determinants are located on the outer capsid proteins VP4 and VP7 [Kapikian et al., 1996].

Only groups A–C have been associated with disease in humans. Group A is the major cause of gastroenteritis in children worldwide. Group B rotaviruses have been documented as causing outbreaks in adults in China [Hung, 1988; Saif et al., 1994]. Group C rotaviruses have been shown to cause both outbreaks and sporadic cases in many parts of the world [Caul et al., 1990; Matsumoto et al., 1989; Oishi et al., 1993].

Routine diagnosis of group A rotaviruses is by ELISA, which detects the group specific antigen. Diagnosis of group C rotavirus infection has been severely restricted as wild type strains have not until recently been grown in cell culture [Shinozaki et al., 1996] and thus reagents are not readily available. Diagnosis is made initially by EM with confirmation by PAGE of dsRNA or by PCR [Gouvea et al., 1990; Jiang et al., 1992; Jiang et al., 1995].

Serological evidence of infection has come from reagents developed for the prototype porcine group C rotavirus "Cowden" strain, which can be serially propagated in cell culture. Recent studies have shown that the Cowden (porcine) rotavirus is distinct from the human group C rotavirus which appears to be a single globally distributed strain [Fielding et al., 1994; Grice et al., 1994; Jiang et al., 1996]. Serological surveys using porcine derived reagents have indicated that anti-

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body to group C rotavirus is present in 3–45% of the human population in certain geographical locations [Caul et al., 1990; Nilsson et al., 1993; Tsunemitsu et al., 1992].

The VP6 gene of human (Bristol) group C rotavirus, carrying the group specific epitopes, has been cloned and sequenced [Cooke et al., 1991]. The purpose of this study was to investigate the seroprevalence of antibodies to human group C rotavirus using recombinant VP6 antigen expressed in insect cells.

MATERIALS AND METHODS

Cells and Viruses

Wild type *Autographa californica* nuclear polyhedrosis virus (AcNPV) and the baculovirus transfer vector pBlueBac2™ were obtained from Invitrogen (Leek, the Netherlands). The virus was propagated at 28°C in *Spodoptera frugiperda* Sf-9 cells in TC-100 medium with 10% foetal calf serum supplement or High Five™ cells (Invitrogen) in serum free ExCell 400™ (JRH Biosciences, Lenexa, Kansas). Recombinant RF strain (group A) VP6 was prepared as previously described [Tosser et al., 1992]. Wild type human group C rotaviruses were cultured in Caco-2 cells, as previously described [Shinozaki et al., 1996].

Construction of Expression Vector

Molecular cloning of human group C VP6 into bacteriophage M13, to produce M13VP6, has been described [Cooke et al., 1991]. To facilitate cloning into pBlueBac2 [Vialard et al., 1990], 2 flanking *Nhe* I sites were introduced into the M13 VP6 recombinant. Briefly, the M13 VP6 RF was digested with *Nsi* I and an *Nsi* I/*Nhe* I adaptor 5′-TGCTAGCATGCA-3′ inserted, yielding M13 VP6 *Nhe* I. Second, M13 VP6 *Nhe*I RF was modified by insertion of an *Xba* I/*Nhe* I adaptor 5′-CTAGAGCTAGCT-3′ at the unique *Xba* I site to allow excision of the VP6 gene as an *Nhe* I cassette.

The VP6 gene was cloned into pBlueBac2 using the unique *Nhe* I site. Recombinants carrying the VP6 gene in the correct orientation with respect to the polyhedrin promoter were identified by PCR with primer pairs (a) 5′-^{4682}ACGTTGAACTCGCCGCAG^{4665}-3′ and 5′-^{4112}CACGTCATGAGGTACATAGT^{4132}-3′, and (b) 5′-^{1220}CTCTAGCTTATCTACAT^{1204}-3′ and 5′-^{124}ATGAATTGATTAGAAC^{140}-3′. The numbers refer to the nucleotide coordinates of pBlueBac2 (a) and the VP6 gene (b). Oligonucleotides were synthesised on a model 381 Automated synthesizer (Applied Biosystems).

Preparation of Recombinant Baculovirus

The pBlueBac2 VP6 recombinant was used to cotransfect insect cells with linearized baculovirus (AcNPV) genomic DNA (Invitrogen) using a cationic liposome mediated transfection method as described by the manufacturer. Amplification of baculovirus and expression of recombinant antigen was performed according to the manufacturer's instructions.

SDS-PAGE Analysis of Infected Cell Polypeptides

Harvested cells were centrifuged and pellets resuspended in 200 μl phosphate-buffered saline (137 mM NaCl; 2.7 mM KCl; 8.1 mM Na_2HPO_4; 1.47 mM KH_2PO_4)(PBS). Pellets and cell supernatant samples were resolved by the SDS-PAGE discontinuous buffer system of Laemmli (1970) with 10% separating and 4% stacking gels. Proteins on the gel were stained with 0.1% PAGE blue 83 (Fluka, UK).

Immunoblot Analysis of Expressed VP6

High Five™ cell culture supernatants collected 96 hours post infection were dialysed against PBS to remove low molecular weight impurities in the cell culture medium. Proteins were resolved by SDS-PAGE and electroblotted onto nitrocellulose (0.8 mA/cm^2 for 1 hour using a Trans-blot semi-dry blotting apparatus) with Bjerrum and Schafer-Neilson buffer containing SDS (48 mM Tris; 39 mM Glycine; 20% Methanol; 1.3 mM SDS). The nitrocellulose membrane was cut into strips for immunostaining, blocked with 5% nonfat dried milk in Tris-buffered saline (20 mM Tris: 500 mM NaCl pH 7.5) and probed with 4 convalescent serum samples (diluted 1:200) from patients known to have been infected recently with group C rotavirus, or hyperimmune rabbit serum at a range of dilutions. Bound antibodies were detected by sequential incubation with anti-species alkaline phosphatase conjugated antibodies and BioRad (Hemel Hempstead, UK) AP stain.

Antibody Production

VP6 harvested from cell culture supernatants was purified by dialysis and used in an immunisation programme to produce hyperimmune sera in "pathogen free" rabbits. Protein (100 μg) in complete Freunds adjuvant followed by 3 boosts of 100 μg protein in incomplete Freunds adjuvant were administered at 10-day intervals to rabbits at 2 subcutaneous sites. The rabbits were bled prior to immunisation and after the third and fourth immunisations.

ELISA Development

A solid phase ELISA was developed to detect serum antibodies to human group C rotavirus VP6. Saturation concentrations of antigen for coating the solid phase was determined using rabbit antibodies. Polyvinyl microtitre plates (ICN Flow) were coated overnight by incubation at 4°C with approximately 250 ng VP6 in 15 mM Na_2CO_3/ 35 mM $NaHCO_3$ pH 9.6 per well. Plates were blocked with 1% nonfat milk in PBS for 30 minutes at 37°C. Sera were screened at 1:100 dilution in PBS containing 1% nonfat milk and incubated at 37°C for 1 hour. After washing with PBS/Tween 20 (0.05%), goat anti-human IgG horseradish peroxidase conjugate (Dako UK Ltd) was added and incubated for 1 hour at 37°C. Tetramethyl benzidine substrate (0.0036% TMB;0.1 M Na acetate; 0.0001% H_2O_2) was added after washing and the reaction stopped with 2M

H_2SO_4 after 5 minutes. The absorbance at 450 nm (A_{450}) was recorded using an automated spectrophotometer (Anthos Labtec). In the absence of documented negative control sera the "cutoff" value was determined as 4 times the A_{450} of a human "control" serum nonreactive with VP6 by immunoblot.

Sera

A collection of 1000 sera grouped by age were collected for the survey over a 6-month period, September 1994 to March 1995, from samples sent to the virology laboratory in Southampton. These included primarily sera from occupational health care workers and antenatal patients sent for routine screening in the 21–60 year age groups. Sera from other age groups were from patients requiring screening mainly for respiratory infections. Ten convalescent sera from proved cases of group A rotavirus infection and 4 from group C rotavirus (Bristol PHL) were available for specificity assays.

Determination of Antibody Specificity of Group A and Group C Convalescent Sera

Serum samples were diluted in PBS containing 1% nonfat milk with or without 10μg/ml of either group A or C recombinant VP6 and incubated for 30 minutes at 37°C. These dilutions were assayed for antibodies to both group A and C rotavirus VP6 by ELISA as described previously.

Immunofluorescence

Caco-2 cells infected with wild-type human group C rotavirus were fixed on glass slides with acetone and incubated with hyperimmune rabbit sera diluted 1:100 in PBS at 37°C for 30 minutes. Bound antibodies were detected by incubation at 37°C for 30 minutes with swine anti rabbit-FITC conjugate (Dako UK Ltd) counterstained with 0.0025% Evans blue.

RESULTS

Isolation and Characterisation of Recombinant VP6

Recombinant baculoviruses carrying the VP6 gene were isolated by serial rounds of single plaque purification in Sf9 cells. VP6 could be detected by SDS-PAGE at 24 hours post infection (p.i.) with maximal synthesis at 72 hours. The VP6 was exported to the cell culture medium beginning at 72 hours p.i. with maximum amounts detected at 96 hours p.i. Infection of High Five™ cells increased the yield of expressed protein and facilitated protein production in a serum free environment (Fig. 1). Recombinant VP6 was harvested from the cell culture medium 96 hours p.i. and dialysed against PBS. The VP6 protein was seen to spontaneously form particles similar to single shelled virions and in addition honeycomb lattice structures were observed (Fig. 2) similar to those described for group A rotavirus [Estes et al., 1987; Hung, 1988; Tosser et al., 1992], confirming it was similar in conformation to native VP6.

Purified recombinant VP6 was used to immunise

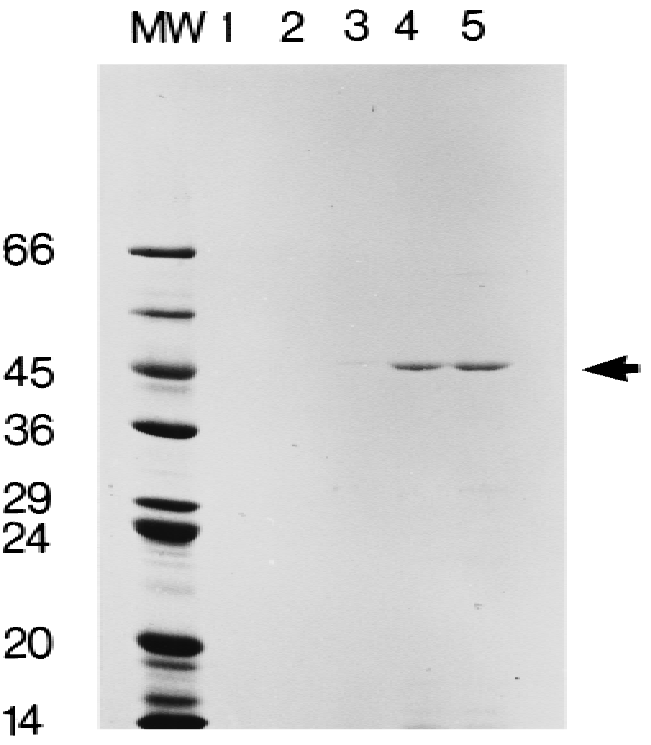

Fig. 1. Time course of human group C rotavirus VP6 expression in insect cells. Cell culture media from High Five™ cells infected with recombinant AcNPV-VP6 were harvested at 24-hour intervals from 24–120 hrs (lanes 1–5) and analysed by SDS-PAGE. The position of VP6 is arrowed to the right. Molecular weight markers (kDa) are labeled to the left.

rabbits and the reactivity of hyperimmune sera was assessed by Western blot using purified VP6 as an antigen. The rabbit sera reacted with VP6 up to a dilution of 1:100,000 (data not shown). Immunofluorescence analysis showed that the rabbit antisera also reacted with wild type human group C rotavirus grown in Caco-2 cells (data not shown). These antibodies were used to optimise an antibody detection ELISA with purified VP6 on the solid phase.

Serological Survey

A collection of 1000 sera, grouped by age, were collected for the survey and screened for antibodies to group C rotavirus at a dilution of 1:100. The mean distribution showed that 43% of sera reacted positively with the greatest numbers in the 71–75 year age group (66.1%). As four sera were from infants aged less than 1 year (2 reactive and 2 nonreactive) these were excluded from the age group calculations as the results could represent maternally derived antibody (Table I). The scattergraph of sample to negative ratios (Fig. 3) shows the antibody distribution to be spread over a wide range of values with no clear distinction between those negative and positive. The assay was designed to detect any antibody to group C rotavirus to determine prevalence and not as a diagnostic assay. The value of the mean S:N ratio of the negative sera plus 3 standard deviations was 4.05.

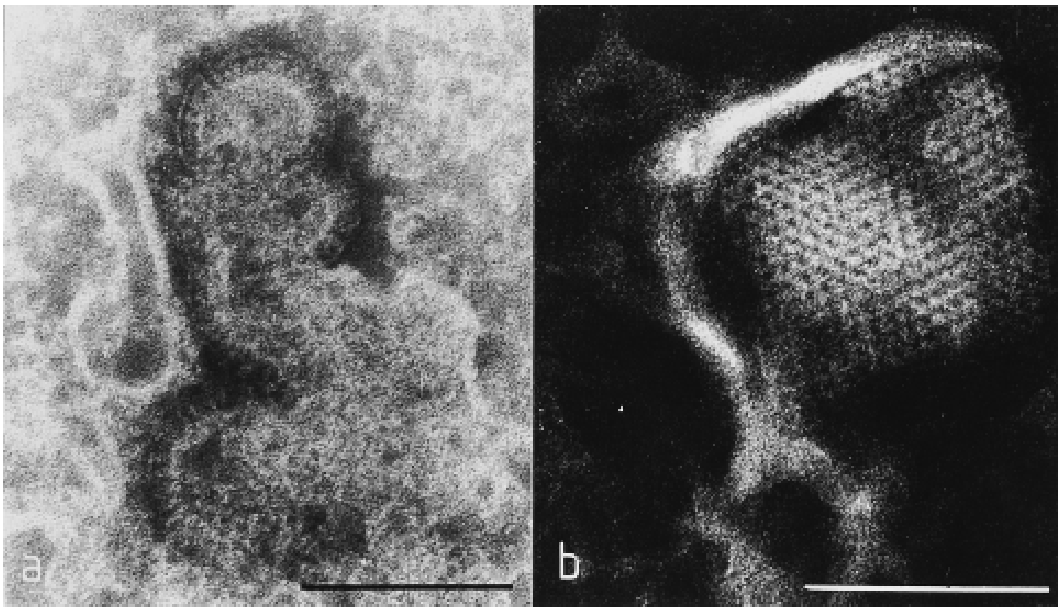

Fig. 2. Spontaneous assembly of baculovirus expressed VP6 into structures with capsomere like morphology. Cell culture media collected 96 hours p.i. from **(a)** Sf 9 cells showed virus-like particles and from **(b)** High Five™ cells showed a honeycomb lattice structure. Cell culture supernatants were adsorbed onto carbon-coated grids, stained with 0.75% phosphotungstic acid, pH 6.0, and visualised using a Hitachi H7000 transmission Electron Microscope. The scale bars equal 100 nm.

TABLE I. Serological Survey for Antibodies to Human Group C Rotavirus VP6

Age range (years)	Number tested	Number positive (S:N > 4)	% positive
1–5	49	13	26.5
6–10	72	22	30.5
11–15	45	9	20
16–20	80	20	25
21–25	64	20	31.3
26–30	61	24	39.3
31–35	72	34	47.2
36–40	53	27	50.9
41–45	64	27	42.2
46–50	61	28	45.9
51–55	63	30	47.6
56–60	62	29	46.7
61–65	66	37	56.1
66–70	60	37	61.7
71–75	62	41	66.1
76+	62	33	53.2
Total	996	431	43.4

A selection of 90 of the sera from children under 10 years and 90 of the sera from adults aged between 31 and 40 years were screened for antibodies to group A rotavirus using recombinant VP6 from the bovine RF strain [Tosser et al., 1992]. In these groups, 85.4% of children and 97% of adults were sero-reactive for group A rotaviruses.

Specificity of the Group A and Group C Antisera

Ten convalescent group A rotavirus sera were screened; 6 were negative, 2 borderline, and 2 positive for group C rotavirus antibodies. The 2 positive sera, 1 of the negative sera, and 4 group C rotavirus convalescent sera were titrated to endpoint with recombinant VP6 from both group A and group C rotaviruses. One group C serum and 2 group A sera showed a significant difference in endpoint dilution (greater than fourfold) in their reactivity to recombinant VP6 of group A and C rotavirus, suggesting a specific response.

To determine whether antibodies in the remaining serum samples were cross-reacting or specific, they were absorbed with recombinant group C VP6. Serum dilutions were incubated with 10 μg/ml group C VP6 and retested with both group A and C rotavirus VP6. A fourfold or greater reduction in endpoint titre was observed for all 4 sera with group C VP6. No reduction in titre was observed with group A VP6. When the sera were absorbed with recombinant group A VP6, a reduction in titre was observed only with the homologous antigen. These results indicate that the sera contained antibodies specific to both group A and C rotaviruses (Fig. 4).

DISCUSSION

Group C rotavirus gene 5 which encodes the major inner capsid protein VP6 was expressed in High Five™ cells with maximum detectable levels exported to the cell culture medium at 96 hours post infection. The recombinant VP6 protein was highly immunogenic in rabbits. These sera were used to develop and optimise an antibody detection ELISA.

In a survey of 1000 sera from all age groups, antibodies to human group C rotavirus were detected by ELISA in 43.4% of the population with a maximum of 66.1% in the 71–75 year age group. These results suggest a higher level of exposure to human group C rota-

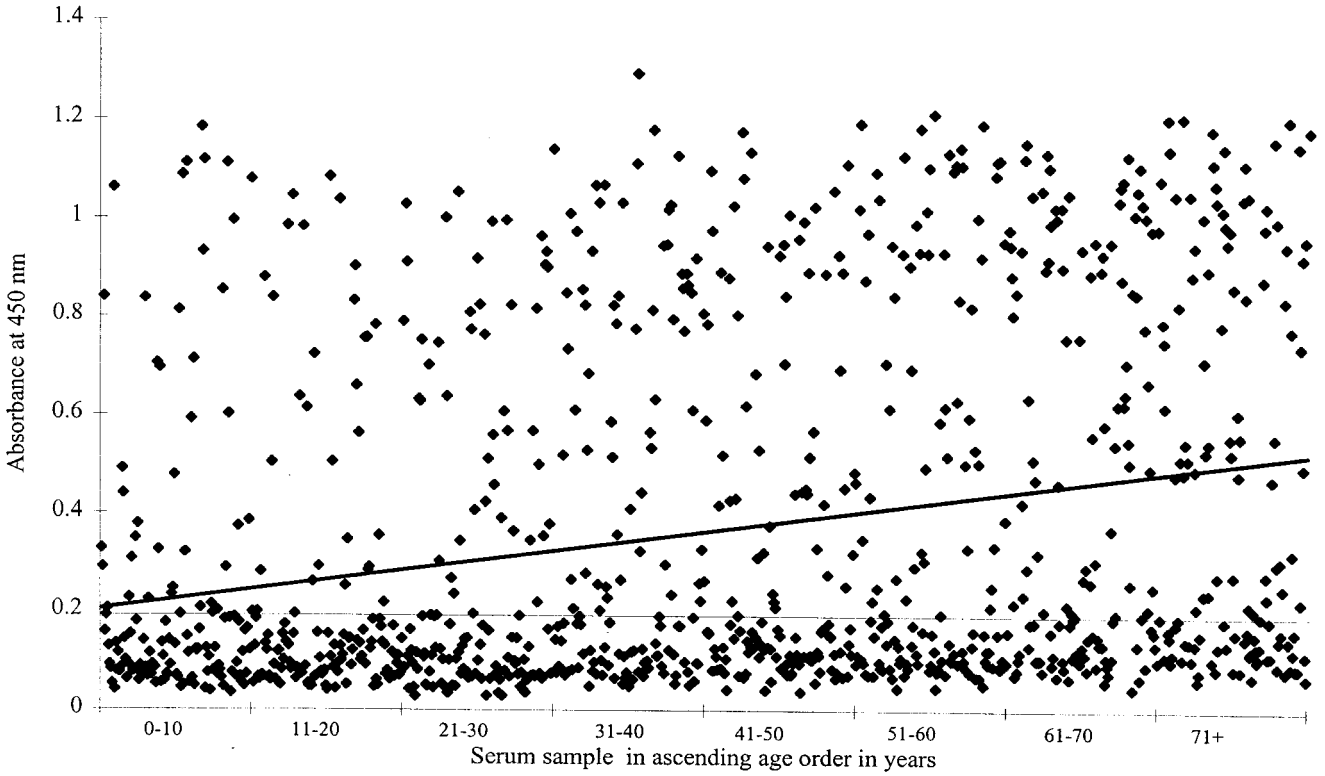

Fig. 3. Epidemiological screen for antibodies to group C rotavirus. 1000 human sera grouped according to age were screened at 1:100 dilution for antibodies to group C rotavirus. Absorbance$_{450}$ was plotted relative to increasing age. Each dot represents an individual serum sample and is plotted at the actual age in years. Sera with an absorbance greater than 0.2 (4× the absorbance of control serum negative by immunoblot), indicated by the fine line, were considered positive. The trendline indicates the increasing antibody with age.

virus than previous studies had suggested using porcine group C rotavirus proteins [Caul et al., 1990; Nilsson et al., 1993; Tsunemitsu et al., 1992].

Tsunemitsi et al. [1992] showed that antigenic cross reactions did not occur between different groups of rotavirus in ELISA, although IF assays using monoclonal antibodies suggested that minor common epitopes exist on the VP6 protein of group A and C rotaviruses. Some cross reactivity was shown to occur with the high titre serum (Group C convalescent serum 3) at 1:100 dilution only. This serum had high levels of antibody to both group A and C rotavirus. The fact that 6 group A convalescent sera were negative for group C antibodies would suggest that this is not a common problem and cross reactive antibodies may exist only in high titre sera from patients exposed to both group A and C rotaviruses. A clear distinction was seen between the proportion of sera that had detectable antibodies to group A rotavirus and group C; if cross reacting antibodies were common, this would not have been observed. The development of an assay using peptides to reactive epitopes specific to the group determinants would resolve this question.

The prevalence of group C rotavirus antibodies was unexpectedly high, considering the low detection rate of group C rotavirus in clinical samples from cases of gastrointestinal disease. This suggests that infection due to group C rotavirus may be milder than that due to group A rotavirus and that infections are not generally investigated. In pigs, high seroconversion rates (58–100%) are observed for group C rotaviruses relative to their actual detection rate. In one study, only 5% of rotaviruses identified from cases of porcine diarrhoea were positive for non group A rotaviruses [Saif et al., 1994], suggesting that subclinical infections may be common in some herds.

Throughout life, humans are repeatedly infected with rotaviruses [Fonteyne et al., 1978]. Symptoms are generally severe only when infants aged between 6 and 24 months acquire a primary infection [Soenarto et al., 1981]. Cross-protection has been demonstrated with heterotypic infection of group A rotavirus [Bridger et al., 1987; Brussow et al., 1988; Feng et al., 1994]. Studies with recombinant outer capsid proteins VP4 and VP7 will help determine whether cross protection between rotavirus groups occurs and whether a prior exposure to group A rotavirus attenuates symptoms of subsequent group C rotavirus infection.

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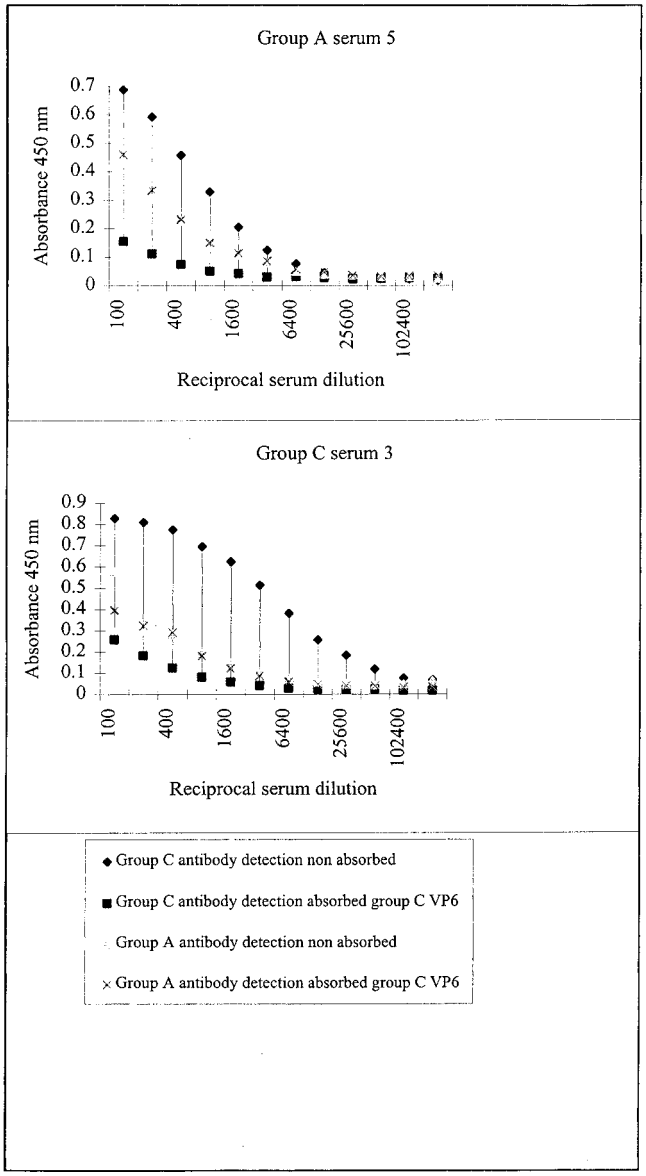

Fig. 4. Representative graphs to show the specificity of the group C rotavirus ELISA. Serum dilutions were absorbed with group C recombinant VP6, titrated to endpoint, and tested by ELISA for antibodies to group A and C rotavirus. **Top:** Convalescent serum, group A serum 5. **Bottom:** Convalescent serum, group C serum 3.

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